CELL LINEAGE TRACING OF OSTEOBLASTS WITHIN THE PERIODONTAL LIGAMENT DURING ORTHODONTIC TOOTH MOVEMENT

A Thesis

by

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ABSTRACT

Background: The periodontal ligament is known to play a role in the bone remodeling response to orthodontic tooth movement and to contribute specifically to osteogenic cell populations involved in this process. Cell lineage tracing is a technique which enables the tracking of specific cell populations throughout differentiation *in vivo*. 3.2kbCol1α1 is present in early osteoblastogenesis and can therefore be used to trace osteoblasts and similar related osteogenic cells. Purpose: The purpose of this study was to a) create a valid murine model of orthodontic tooth movement and, b) orthodontically treat temporally inducible cell lineage tracing mice to study osteoblasts response within the periodontal ligament. Materials and methods: 3.2Col1α1 mice were used in this study. Orthodontic appliances were bonded between the maxillary first molar and the maxillary incisors to induce mesial movement of the maxillary first molar. The mesial root of the maxillary first molar was used for evaluation in this study. 5 groups of mice were studied with 4 mice in each group; group 1: no induction, no orthodontic treatment; group 2: induction, no orthodontic treatment; group 3: induction, 1-day orthodontic treatment; group 4: induction, 2-day orthodontic treatment; group 5: induction, 4-day orthodontic treatment. Radiological analysis was performed. Angle of first molar was quantified to determine presence of tooth movement. Mean widths of periodontal ligament were compared. 3.2Col1α1 signal was

characterized. H&E staining and anti-Ki67 and anti-CathepsinK immunofluorescent staining was performed. Results: Orthodontic treatment induced orthodontic tooth movement. 3.2kbCol1α1-positive cells were identified within the periodontium. The number of 3.2kbCol1α1-positive cells increased in response to orthodontic treatment. Ki67 expression was inversely related to length of orthodontic treatment. CathepsinK activity was inconclusive regarding the determination of a relationship between 3.2kbCol1α1-positive osteoblast patterning and osteoclast activity. Conclusions: 3.2kbCol1α1-positive cells are present in the periodontal ligament and increase in response to orthodontic tooth movement.

To H.C.M.

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Introduction

In 1888, L.E. Custer published a paper *Intermittent Pressure: Its Relation to Orthodontia*. 130 years later, orthodontists are still scrutinizing the interdependence of force and movement and how it complements craniofacial anatomy[1]. Orthodontic treatment is the process by which force is applied in order to induce the remodeling of the craniofacial complexes. While the gross outcomes of orthodontic treatment are well documented, less is known about the molecular interactions that take place during orthodontic treatment[2]. To be able to precisely analyze and manipulate orthodontic treatments and forces, it is necessary to conduct prospective and *in vivo* studies to accurately test and manipulate experimental variables[3]. Due to the preservative and restorative nature of orthodontic treatment, it is often difficult to find samples undergoing orthodontic treatment available for analysis. In addition, only recently has basic science been incorporated into orthodontic treatment planning considerations[22]. Currently, ~13% of orthodontic research publications consists of case studies, which are not scientifically rigorous enough to affect future treatment guidelines. The need to address this bench to bedside dilemma in orthodontics is apparent— only then can L.E. Custer's original inquisition be meaningfully pursued.

Animal models provide the alternative to human subjects in cases where ethics, number of samples, or transgenic constructs make human experimentation impossible[4]. Even though animal models of orthodontics have been utilized, there is currently no standard orthodontic tooth movement animal model that may be used as baseline for preliminary or pilot studies. This results in studies using various animal sizes, species, experimental designs. This study's purpose is to use a temporally inducible cell lineage tracing mouse model to create an orthodontic tooth movement animal model for studying osteoblasts within the periodontal ligament in response to orthodontic treatment. This research will, for the first time, trace 3.2kbCol1α1-positive osteoblast during orthodontic tooth movement.

The forthcoming literature review will provide the background necessary to understand the basis of both the need for and the research theories underlying this study. Chapter II will expand on the reasoning supporting the design of the study based upon the literature review; chapter III will provide methods utilized in this study; chapters IV – VII will review results, conclusions, discussion and future studies.

Literature Review

Three distinct tissue types are directly involved in orthodontic movement: bone, periodontal ligament, and dental tissue[22]. Current knowledge of these tissues' response to orthodontic treatment are mostly

understood as they relate to themselves, not as they relate to each other or as they interact during orthodontic treatment.

The Periodontal Ligament

Teeth are embedded within alveolar bone. Lining the tooth sulcus is the lamina dura, a plate of cortical bone. The alveolar crest is the region of alveolar bone between teeth that extends to the cementoenamel junction. The periodontal ligament (PDL) is a fibrous structure which anchors teeth by connecting cementum to alveolar bone[23]. The PDL is predominantly composed of collagen type I. These connections form a three-dimensional supportive structure around roots. The portion of the PDL that inserts into the cementum or alveolar bone is known as Sharpey's fibers. The PDL is composed of 5 types of principal fibers: alveolar crest fibers, horizontal fibers, oblique fibers, periapical fibers and interradicular fibers. The directionality of these fibers allows the PDL to not only anchor from cementum to bone, but also enables the PDL to act as a robust shock absorber. The PDL is capable of absorbing light forces through the compression of its vascular fluids[5], while heavy forces are absorbed by the principal fibers. The PDL also serves important roles in sensory functions, remodeling, and provides a cell source for surrounding tissues.

During orthodontic tooth movement the PDL, as well as surrounding bone, is remodeled. It is well understood, in fact, that the

alveolar bone remodeling observed in orthodontic tooth movement occurs as a response to the stress patterns experienced by the PDL. Principal fibers undergoing remodeling characteristically become disorganized and lose their directionality. The vascular components in the PDL on the pressure side of orthodontic movement experience compression, noted by the constriction of blood vessels. The opposite occurs on the tension side, where blood vessels notably experience dilation during orthodontic treatment.

The PDL is believed to host undifferentiated ectomesenchymal cells that are capable of maintaining PDL, cementum, and bone[6]. These three tissues experience different lifespans of development and remodeling: the PDL is constantly in flux, responding to stimuli and maintaining itself; bone undergoes comparatively slow but constant remodeling throughout life; after initial modeling, cementum undergoes extremely limited remodeling during normal homeostasis. Cementum can be resorbed but does not experience balanced and programmed remodeling similar to bone[7]. The PDL contributes to the differentiation of cementoblasts and osteoblasts, but the mechanotransduction pathway that induces this process in orthodontic tooth movement is not well understood[24, 25, 26].

Bone Homeostasis, Remodeling, and Bending

There are three major cells that contribute to bone homeostasis: osteoblasts, osteocytes, and osteoclasts. Osteoblasts secrete matrix that allows for the building of bone. Osteocytes are bone cells that maintain bone tissue and remain active for up to years. Osteoclasts are multinucleated cells that are responsible for the resorption of bone. Osteoblasts and osteocytes are of the same ectomesenchymal lineage, as some osteocytes are believed to be mature osteoblasts trapped within mineralized bone matrix, while osteoclasts are derived from hematopoietic stem cells. Osteoblasts and osteoclasts are able to regulate each other in order to maintain bone homeostasis. Osteoblasts secrete receptor activator of nuclear factor kappa-B ligand (RANKL) which is bound by the surface-bound receptor activator of nuclear factor kappa-B (RANK) protein of osteoclasts. The binding of RANKL will stimulate osteoclastogenesis. Osteoblast secretion of osteoprotegrin (OPG), a RANK antagonist, allows osteoblasts to bidirectionally regulate osteoclast activity. Additionally, osteoblasts secrete macrophage colony stimulating factor (m-csf), which serves a role in osteoclast differentiation. These synchronized molecular mechanisms between osteoblasts and osteoclasts allow for the balance of bone deposition and resorption to be maintained.

Normal remodeling consists of removing old bone and replacing it with new. When external forces are applied, a more rapid and acute activation of remodeling can be observed. In orthodontic movement, the

remodeling process occurs to allow for alveolar bone to accommodate the new external forces, the structure's response to the new function. Bone will be resorbed in areas of pressure by osteoclasts, and matrix will be secreted in areas of tension by osteoblasts (see figure 1.). "Uneven" remodeling will allow the craniofacial complex to compensate for the new discrepancy in forces, creating new sites of bone apposition and deposition.

Bone bending is a separate process by which bone itself is flexible enough to "bend" in response to force rather than remodel before any tooth movement will occur[8]. Both remodeling and bone bending can be occurring at the same time. Bending however would skew the dispersion of pressure-tension forces and potentially change the areas undergoing remodeling. Additionally, bone bending does not predictably occur in the direction of movement. While the presence of bending or non-remodeling movement has been observed, it is not well understood.

Cell Lineage Tracing

Cell lineage tracing, or fate mapping, is a process by which a cell is permanently labeled thereby labeling the cell's progeny as well. When one cell type or group of cells are targeted and subsequently labeled, cell lineage tracing can indicate differentiation process that occurs throughout growth and development, a niche of pluripotent cells, or the spacial and temporal patterning of the progeny[9]. Because collagen I is a marker in

early osteoblastogenesis, targeting a collagen I promoter as a construct for cell lineage tracing enables the study of bone modeling and remodeling events beginning with early osteoblasts.

Inducible Cre-Lox transgenic mouse systems are available which allow for temporal activations of gene-of-interest, like Cre-Estrogen Receptor (ER) systems. These systems are able to remove LoxP sites, which flank stop cassettes or genes of interest. If a stop cassette is preceded by a promoter of interest and followed by a reporter gene, then the removal of the stop cassette will enable the expression of that promoter to be traced. Reporter genes are inserted into regulatory or conserved gene loci, allowing the activation to be heritable. If the promoter is cell-type specific, then the design enables tracing of tissue-specific cell-populations. For example, osteoblast specific promoters include 2.3Col1α1, 3.2Col1α1, 3.6Col1α1 and Osx/Sp7. A transgenic mouse line with a promoter coupled to a reporter gene like green fluorescent protein (GFP), or cyan fluorescent protein (CFP) allows for cells to be visually traced,

Tamoxifen inducible cre-lox systems are currently available in two generations: Cre-ER^T and Cre-ER^{T2}. A higher dose of tamoxifen is required to induce a Cre-ER^T system than a Cre-ER^{T2} system. Tamoxifen is a selective estrogen receptor modulator, meaning that it can act as both an agonist and an antagonist in a tissue dependent manner[10]. The mechanism of tamoxifen's uptake into a cell is not understood, but is

hypothesized to be similar to steroid membrane diffusion, since the structure closely resembles estrogen[11]. Estrogen has been noted to inhibit bone resorption, and tamoxifen has been noted to induce similar effects in bone.

Collagen I Promoter Murine Models

Generating a standardized model of orthodontic tooth movement that can be easily replicated and edited for individual study designs, researchers will reduce redundancy, increase statistical strength of results, and produce more meaningful translational research outcomes. Animal models provide a solution for orthodontic researchers, as a) *in vivo* samples of all three tissues may be obtained, b) samples may be obtained during orthodontic treatment, and c) study time points may be planned prospectively. Mice are the most commonly used animal in research as they are easily generated and maintained, the majority of human genes are found within mice, and the transgenic mouse lines available for in-depth targeting of specific cell populations[12]. Even though mice are a popular animal model choice, selection of the appropriate murine model is paramount for the success and validity of a study[13]. Osteoblast-specific models available include promoter regions targeting Runx2, Osterix, Col1α1, and Osteocalcin. Within Col1α1 promoter classes, 2.3kbCol1α1, 3.2kbCol1α1 and 3.6Col1α1, and Col1α2 models exist[14].

Collagen type I, made up of two pro-α1(I) chains and one pro-α2(I) chain, is a fibrillar collagen present in the extracellular matrix and the most abundant of the collagens. Pro-α1(I) is produced by the COL1A1 gene and pro-α2(I) is produced by the COL1A2 gene in humans[15]. It is a structural protein that plays a support role in many connective and skeletal tissues like cartilage, tendon, skin, tooth, dentin, and bone and therefore is secreted by fibroblasts, odontoblasts, cementoblasts, and osteoblasts, making it a useful molecular marker in orthopedic research. Osteoblasts secrete matrix necessary for bone, known as osteoid. The osteoid is rich in molecules instrumental for bone formation including osteocalcin, alkaline phosphate, and collagen I. Because of the large amounts of type I collagen present in the osteoid secreted by osteoblasts, it is a highly sensitive indicator of osteoblast activity[16]. Collagen I itself is therefore not only a product of the process of "bone building" but is an essential element of proper osteogenesis and overall bone organization[17]. Transgenic mouse models that target collagen I promoter or enhancer activity can therefore be used as a reliable marker of various population and stages of osteoblasts and offer *in vivo*, endogenous indicators of both the upstream and downstream effects of external experimental manipulation.

2.3Col1α1 is characterized by expression of positively labeled cells in odontoblasts and mature osteoblasts[18]. These lineage tracing mouse models are available in both inducible and non-inducible constructs. For example, a noninducible transgene enhanced green fluorescent protein

(EGFP) 2.3Col1α1 mouse should exhibit fluorescence in collagen I cells *de novo*. These models are useful to those researchers interested in studying early time points who are worried about the toxicity or interference of a drug-induced model or who want to combine tracing methods with another cre-inducible line.

3.2Col1α1 is similar in its expression to 2.3Col1α1, in that both target the alpha 1 chain of type I, but use different upstream promoter regions to do so. 3.2Col1α1 is expressed earlier in osteoblastogenesis and osteoblast progenitor cells when compared to 2.3Col1α1, whose expression peaks in mature osteoblasts[19]. 3.2Col1α1 is available in both 1st and 2nd generation inducible constructs. While its use is not as widespread as 2.3Col1α1, 3.2's more precise nature makes it particularly suited for orthopedic as well as mesenchymal stem cell research. Well-suited to study osteoblast maturation itself, 3.2Col1α1 is a better selection when the researcher is concerned with detecting even the smallest changes within distinct osteoblast populations.

Mice with a 3.6Col1α1 inducible construct are now available, a promoter that was until recently only available in rats[19]. This transgene was transferred into mice, resulting in a noninducible model whose positive cells are expressed in mature collagen I tissues. For example, Jackson Laboratories has reported their pOBCol3.6GFPcyan mouse model expresses positive cells on the surface of bones at 3-5 months. This is a design that can be useful not in its specificity but in its exclusion,

and is a model worth considering for those whose cell lineage tracing experiments span into mouse adulthood. 3.6Col1α1 expression has been reported in all collagen I positive tissue types, making it a versatile model for those working in ectodermal and epithelial research[14]. The cyan reporter is weaker than EGFP however, so this should be taken into account when selecting a 3.6 model against a 2.3 model.

Col1α2 is a construct that focuses on the α2 chain of collagen 1, and whose expression varies greatly from that of the α1 chain. Col1α2's expression is characterized by positive cells secreted by fibroblasts[20]. While Col1α1's expression is in the osteoblast and odontoblast lineage, Col1α2 provides an avenue to trace the third cell potentiality of collagen type I. Both noninducible and inducible models have been created, allowing for a range of cell lineage tracing designs to occur.

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CHAPTER II

EXPERIMENTAL RATIONALE AND DESIGN

Animal Model Selection

Previous unpublished data utilized a similar orthodontic appliance technique in mice yet utilized an inducible Gli-1 Cre^{ERT2} line over longer (1-2 month) time periods. This resulted in data that was essentially unusable and unable to interpret in a meaningful way-- since Gli-1 has been recognized as an early mesenchymal progenitor marker[1] that identifies both osteogenic and chondrogenic cell lineages, this design resulted in tdTomato-tagged cells heavily distributed throughout the samples. This preliminary data proved to be instrumental in the development of the timeline of this project both in appliance and induction times and in the selection of an appropriate and more selective osteogenic cell marker.

Study Model

In this study, the determination was made to use a 3.2kbCol1α1 promoter based on its highly robust and specific expression beginning in early osteoblasts[2]. $CreER^{T2}$ is a tamoxifen inducible, cre-recombinase driven model that was combined with a ROSA26tdTomato reporter mouse line in this study. A tamoxifen induction will allow cells with an active 3.2Col1α1 promoter to fluoresce with tdTomato, a red fluorescent protein. Because this is an ER^{T2} model, it requires substantially less tamoxifen to

induce than previous models and should therefore affect endogenous bone homeostasis less[3]. Therefore, a 3.2kbCol1α1CreER^{T2}; R26RtdTomato murine model offers a sensitive osteogenic-specific promoter that is well established in osteoblastogenesis combined with a robust fluorescent marker.

Orthodontic Appliances

Currently, there is a clear lack of "industry standard" when it comes to orthodontic movement protocols in mouse models. Current studies vary greatly in their techniques and provide little basis for their rationale for selecting their protocol. In this research, before I sought to begin manipulating variables, it became clear that it was necessary to clearly define a mouse model of orthodontic movement based on basic science, clinical relevance, and practicality in consideration of reproducibility.

One of the most comprehensive developments in the field of establishing a valid mouse model of orthodontic tooth movement was conducted in 2009 by Viecilli et. al[4]. In this study, finite element stress analyses were conducted on a maxillary first molar. They used custom closed coil, 0.003-in superelastic nickel titanium wire springs with 0.19-in lumens, from G&H Wire (Greenwood, Indiana). They used 3mm of this spring to obtain a 0.03-N force with 2mm of activation in their model. The springs were attached between the mice's maxillary first molars and

incisors using ligature wires and resin composite on the incisors (see figure 3.).

While this research is undoubtedly the best thus far, and is largely the basis for my orthodontic protocol, there are improvements that I deemed necessary. I did replicate the type and length of spring used but altered the bonding method. Ligature wires on a mouse model seem wholly impractical— a first molar crown on a mouse is approximately 1mm in length. While not an impossible procedure, the hope of meaningful replication without significant tissue irritation is slim. I therefore decided to forego ligature attachment of the springs and used resin composite alone on both bonding surfaces. Furthermore, as indicated in images published by Viecilli et. al., the springs seem to rest on the buccal side of the maxillary first molar. Because of the force applied to the tooth, this would result in rotating the tooth lingually. Because of this, I chose to bond my springs on the mesial surface of the crown. The spring covers the entire mesial surface evenly. By bonding the spring to the mesial surface of the first molar and the lingual surface of the incisors, I was able to form a similar 8° angle of bond as Viecilli et. al. This bonding method proved to be very reproducible, disturbs none of the other teeth with gingival irritation or mechanical forces, and uses significantly less composite on the incisors when compared to Viecilli et. al.

Timeline Design

Initially, this study intended to bond appliances and induce at the same time. After collection of preliminary samples and subsequent analysis, it became apparent that comparing samples with different lengths of induction would severely limit the strength of the study, as results could not be concluded definitively to be a product of the orthodontic tooth movement. Therefore, I developed a protocol that would manipulate only one variable-- the length of time of orthodontic tooth movement-- so that any difference can be concluded to be a result of orthodontic tooth movement.

Histological and Antibody Selection

Histology

Hematoxylin and eosin (H&E) staining was utilized on each of the samples in this study individually. H&E staining is the most widely used histological staining method and is a fast and convenient way to identify gross histological morphology[5]. Hematoxylin is a nuclear stain, while eosin will stain proteins nonspecifically. This stain is useful in this study for observing the changes overall in the sample morphology, as well as observing the cementum. Because root resorption and cementum remodeling is an observed potential effect of orthodontic tooth movement, histological analysis of orthodontically treated samples is necessary for orientation as well as analysis[6].

Antibodies

Samples were processed with anti- Ki67, and -CathepsinK antibodies. Ki67 is a cell proliferation protein that is expressed through all stages of the cell cycle except for G0[7]. By comparing samples' tomato-positive numbers to intensity of Ki67, we can determine if any change in levels of tomato-positive cells is due to increased specific 3.2Col1 expression or due to increased overall cell proliferation. While the same can be achieved through a cell of interest to total cell count ratio, a cell proliferation intensity ratio is more precise. Ki67 is also preferable to BrdU and EdU because there is no interference with the in vitro model due to additional drug administration. CathepsinK is a protease that is involved in bone resorption and is used as a marker for osteoclast activity[8]. Observing the changes in resorption and osteoclast activity in this model is applicable in order to compare and contrast to osteoblast activity.

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CHAPTER III

MATERIALS AND METHODS

Animals

5 groups of mice were evaluated: control, induction only, 1-day orthodontic tooth movement (OTM), 2-day OTM, and 4-day OTM. 3.2kbCol1α1CreER^{T2}; R26RtdTomato C57/B6 mice were generated and maintained for use in this study. Genotypes of the mice were determined by PCR analysis of genomic DNA extracted from tissue biopsies before postnatal 10 days of age. Pups were weaned at 21 days of age, when they were separated by sex to prevent both pregnancy and aggression. Mice were kept in their weaning groups for the remainder of the study. Mice at 6 weeks of age were used in the study; tamoxifen was administered via intraperitoneal injection on day 39 and mice were sacrificed on day 45. A one-time 100μL dose of 20 mg/mL tamoxifen/corn oil solution was used for induction. Mice were sacrificed through the use of CO_2 asphyxiation. All animal studies were approved by the Institutional Animal Care and Use Committee of Texas A&M University College of Dentistry.

Experimental Treatment

Three time points for orthodontic treatment were tested, in addition to induction-only and null-control groups. Orthodontic treatment time

points were 1-day, 2-days, or 4-days. The total time of reporter gene induction in all time points was 6 days; therefore, in the 1-day treatment group springs were mounted 1-day before sacrifice and five days after induction, in the 2-day treatment group springs were mounted 2 days before sacrifice and 4 days after induction, and in the 4 day treatment group springs were mounted 4 days before sacrifice and 2 days after induction. All animals were sacrificed on the seventh day (see table 1).

Mice were anesthetized with a ketamine/xylazine cocktail at a dose of 0.1mL/20g, administered via intraperitoneal injection. Mice were situated in a prone position with custom designed retractors allowing for access to the maxillary areas. The mesial surface of the first molar was prepped through the use of a water wipe, followed by a 70% ethanol wipe, followed by forced air drying. The surface was then treated with Transbond™ Plus Self Etching Primer, applied according to manufacturer's instructions. A 3mm piece of spring was bonded to the prepared surface using Transbond™ XT Light Cure Adhesive and cured for a total of 20 seconds to ensure set. Orthodontic springs were obtained to generate 0.03 N, ~ 3 grams (G&H Orthodontics, Franklin, IN). The same procedure was then repeated on the lingual surfaces of both incisors, resulting in the spring bonded to the incisors as a unit (see figure 4.).

After treatment, mice were kept warm on a surgical warming mat until conscious. Mice were transferred back to home cages, were they were maintained on DietGel® 31M (ClearH2O, Westbrook, Maine).

Tissue Preparation

Immediately following sacrifice, tissues were harvested and immersion fixed in 4% paraformaldehyde at 4°C, overnight, and decalcified in 10% EDTA. After radiological imaging and in anticipation of frozen sectioning, samples were transferred to 30% sucrose solution overnight. Next, samples were embedded in Tissue-Tek® O.C.T. Compound, (Sakura® Finetek), and sectioned with a Leica CM1850 Cryostat, (Leica Biosystems Inc., Buffalo Grove, IL 60089). 10µm sections were obtained at -26°C on charged slides. Samples were stored at -20°C until use.

Radiographs

Radiological ratios were used to quantify tooth movement. Maxilla were removed from samples and soft tissue was dissected away and hemisected. Samples were then arranged onto the flat surface of a Faxitron model MX-20 Specimen Radiography System (Faxitron X-Ray Corp., Lincolnshire, IL, USA). Analysis of radiographs was performed using ImageJ software. The angle of the first molar was measured, using the buccal cusp, the apex of the mesial root and the apex of the distal root

to form the angle. This measurement of tooth movement indicates change from the control angle as a signal in tooth movement, as we know that bodily movement is not possible with this method of force application. This form of measurement also reduces error which is produced as a result of angle irregularities during image capture.

Histology

Samples were stained with hematoxylin and eosin, and alcian blue with nuclear fast red counterstain, separately. Frozen sections stored at -20C were brought to rest at room temperature for 20 minutes before beginning the staining protocols, Samples were mounted with Permount™ mounting medium (Electron Microscopy Sciences, Hatfield, PA) and stored in the dark at room temperature.

Immunofluorescence

Immunostaining was performed as previously described[1], using 5% BSA in PBS as blocking solution and 1% BSA in PBS for antibody dilution. Samples were stained with rabbit anti-collagen I antibody (Abcam; 1:100), rabbit anti-DMP1 antibody (provided by Dr. Chunlin Qin at Texas A&M University, 1:400), rabbit anti-Cathepsin K (Abcam, 1:100) and Ki67(Invitrogen, 1:100). Corresponding AlexaFlour-488 secondary antibodies (Thermofisher, 1:1000) were used to enable fluorescent detection.

Microscopy

Scanning light microscopy was utilized to image histological samples. Confocal microscopy was used to capture fluorescent images with a SP5 Leica microscope. Red color was detected at 581nm, green at 488nm, and blue at 405nm (UV). Images were taken using the stack function at the frequency of 100Hz with a resolution of 1024x1024.

Sample Analysis

All quantitative analyses of images, including radiographs, was conducted using ImageJ software[2]. To calibrate the scale of radiological images, an image was taken of a 6mm OrthoEasy® pin (Pforzheim,Germany) shooting from the apex of the pin. This end-on radiograph allowed for the widest part of the pin to be imaged accurately

Angles of the first molar were measured on radiographs using the mesio-buccal crown cusp point, the mesio-superior point of the mesial root of the first molar, and the mesio-superior point of the mesial root of the first molar The apex of the angle is formed at the mesio-superior point of the mesial root of the first molar (see figure 7). This angle was measure on 4 samples each from control, tamoxifen, 1-day, 2-day, and 4-day groups.

Width of the periodontal ligament (PDL) space was measured by obtaining measurements from histological slides. Images of the mesial root of the first molar were oriented in a way the expected quadrants from tipping forces (see figure 2.) could be superimposed over the root, with the

y-axis parallel and through the dental pulp and the x-axis bisecting the root from furcation to apex. 6 measurements of width, alveolar-PDL junction to PDL-cementum junction were obtained in each quadrant. 4 samples each from control, tamoxifen, 1-day, 2-day, and 4-day groups were measured.

Statistical Evaluation

Student's t-tests were used to compare means of angles. Mean and standard deviation were used to characterize mean width data. Because distances from histologically processed samples should only be considered semi-quantitative, statistical test for differences was not performed.

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CHAPTER IV

RESULTS

Radiological Analysis

Qualitative analysis indicates that there is no perceptible tooth movement or difference between control or tamoxifen samples (see figure 6). There is space observed in the 1-day, 2-day, and 4-day orthodontic treatment samples. Artifact from remaining composite resin on the first molar results in areas of radiolucency on the crown on the first molar on some samples. Comparing the orthodontically treated samples to the control samples, there is space between the second molar mesial surface and the first molar distal surface that is not observed in the controls. It is not apparent between orthodontic time points if the distance of orthodontic movement is significantly different.

Quantitative analysis results are displayed in figures 8-9. Using student's t-test to compare the means of control and tamoxifen first molar angles, the resulting p-value of 0.402 indicates that there is no significant difference between these two groups. A one sample t-test was utilized to compare the orthodontic treatment group means to the tamoxifen control value of 45.62 (mean in degrees) individually. Each p value was <0.05.

Histological Evaluation

Qualitative analysis of H&E stained samples showed: 1) periodontal ligament fiber reorganization when comparing orthodontic treated samples to the control, 2) increased secretion of acidic matrix with increased length of orthodontic treatment and more matrix when compared to the control, 3) presence of osteoclasts with large resorption bays in the 4-day treatment group (see figures 10, 11).

Quantitative results of mean periodontal space width in four quadrants are illustrated in figure 12. No statistical analysis was performed. In the pressure root area, there was a sharp decrease in the mean width at 1-day of treatment, with an increase in width nearing control at 4 days of treatment. In the tension root area, there was little overall change in the mean PDL mean width between control and any treatment groups. In the pressure crown area, the 4 day group showed a noticeable decrease in mean PDL width. Finally, the tension crown quadrant mean PDL width steadily decreased as treatment time increased.

Cell Lineage Tracing

3.2Col1α1 Signal

3.2Col1α1-positive cells were detected in the dental pulp, cementoblasts, Tomes' fibers, odontoblasts, and osteoblasts (see figure 13). Qualitative analysis of the difference in signal between the control and orthodontically treated samples shows a noticeable increase in the

number of labeled cells. Very noticeable is the increased labeling in the odontoblast processes in response to orthodontic movement (figures 14, 15). The number of labeled cells in the PDL space is markedly increased in all orthodontically treated samples when compared to the control, but it is not apparent if there is a difference between orthodontically treated samples in number of positive cells.

Patterning

3.2Col1α1-positive labeled cells are not evenly distributed throughout the PDL space in orthodontically treated samples. Overall, there is a greater concentration of cells in the mesial PDL space when compared to the distal PDL. There is an increase in positive cells in the PDL in the crown pressure area of orthodontic treated samples when compared to the control. Additionally, the 4-day sample exhibited a striking labeling of the odontoblast processes that clearly extends from the odontoblasts lining the dental pulp space to the dento-cemental junction.

When comparing the root sections of samples (see figure 16), there was increased presence of positive cells in the cellular cementum when comparing the control to 1-day. In the 1-day sample, there were labeled cells present lining the cementum that was not observed in the control. Comparing the 1 to 2-day sample, there was a drastic increase in labeling in the day 2 sample. Between the day 2 and day 4 samples, the labeling increased further. Positively labeled cells were present in the cellular cementum, the dento-cemental junction, the PDL space, the PDL-alveolar

junction, alveolar bone, and the bone marrow spaces, in addition to the aforementioned dental pulp and odontoblasts. The shape of the PDL space in the 4 day sample is similar to the shape of the PDL in the control, but the width of the PDL is different: wider on the tension side and thinner on the pressure side.

Examining the crown tension PDL space of samples do not show a large difference in the labeling between the control, 1-day, 2-day, or 4-day groups (figure 15).

Immunoflourescence

Ki67

(See figures 17-19). Comparison of 1-day, 2-day, and 4-day samples show overall decreased expression with increased length of orthodontic treatment. This decrease in Ki67 was observed in the PDL space, the alveolar bone, and the bone marrow. Comparing quadrants of the same sample showed a difference in expression in the PDL depending on the area. Areas expected to experience tension exhibited more Ki67 activity, while pressure areas exhibited markedly less.

CathepsinK

(See figure 20). CathepsinK expression strongly decreased in response to length of orthodontic treatment. Expression was detection in the alveolar bone, the dental pulp, and the PDL space. While osteoclast activity was determined to be the strongest at day 4 in HE stained

sections, CathepsinK activity in the day 4 samples was not as strong as in day 1 samples.

CHAPTER V

DISCUSSION

Tamoxifen

In cre-lox constructs, tamoxifen is commonly referred to as an estrogen receptor (ER) antagonist[1,2]. This is incorrect and is fundamental flaw in the understanding of tamoxifen. Tamoxifen is a selective estrogen receptor modulator (SERM). SERMs are able to act as both ER agonists and antagonists, dependent on tissue type. Of particular importance to this research, SERMs, especially tamoxifen, are known to interfere with normal bone homeostasis[4].

Tamoxifen is commonly administered in conjunction with chemotherapy regimens in humans[5]. In addition, SERMs have been approved for the treatment of postmenopausal women[6]. It is from these types of studies that it is well known that SERMs inhibit osteoclast differentiation, and even increasing osteoblast activity. While the mechanism of this class of drugs has been a breakthrough for the treatment of osteoporosis and osteoarthiritis, it is detrimental to orthopedic research depending upon tamoxifen-inducible cre-lox models. In this study, if the physiological response of bone remodeling is inhibited, the premise of the study is invalid.

Using imperfect transgenic models may be necessary currently, but researchers should be aware of the risks—rigorous dose-response preliminary studies should be performed to ensure that a phenotype is not

produced from tamoxifen induction alone. While this study attempted to control for this mechanism of tamoxifen, using an SERM in orthopedic research at all severely limits the validity and applicability of this research.

Doxycycline-inducible models are not a solution, but may be a viable alternative depending on the target cell population. For osteoblast-specific targeting, there is an Osx/Sp7 doxycycline inducible model available from Jackson Laboratories (stock number 006361, Bar Harbor, ME).

CathepsinK

CathepsinK results were anomalous in that expression was noted in soft tissue. CathepsinK has previously been reported to be a highly specific osteoclast marker. Upon further research, I found that CathepsinK is present in many craniofacial tissues, including the dental pulp and periodontal ligament[8]. Upon the realization of this finding, it is apparent that CathepsinK is a poor choice for an osteoclast-specific marker in the study of tooth movement. In the future, the use of anti-tartrate-resistant acid phosphatase (TRAP) antibody would prevent similar results.

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CHAPTER VI

CONCLUSIONS

1. The methods and techniques described in this study produce a valid and replicable murine model of orthodontic tooth movement.
2. 3.2Col1α1 positive cells are present in the periodontal ligament and respond to orthodontic treatment.
3. 3.2Col1α1 positive cells proliferate in response to orthodontic treatment.
4. Dentin responds to orthodontic force, evidenced by increased 3.2Col1α1 labeling in odontoblasts.

APPENDIX A

FIGURES

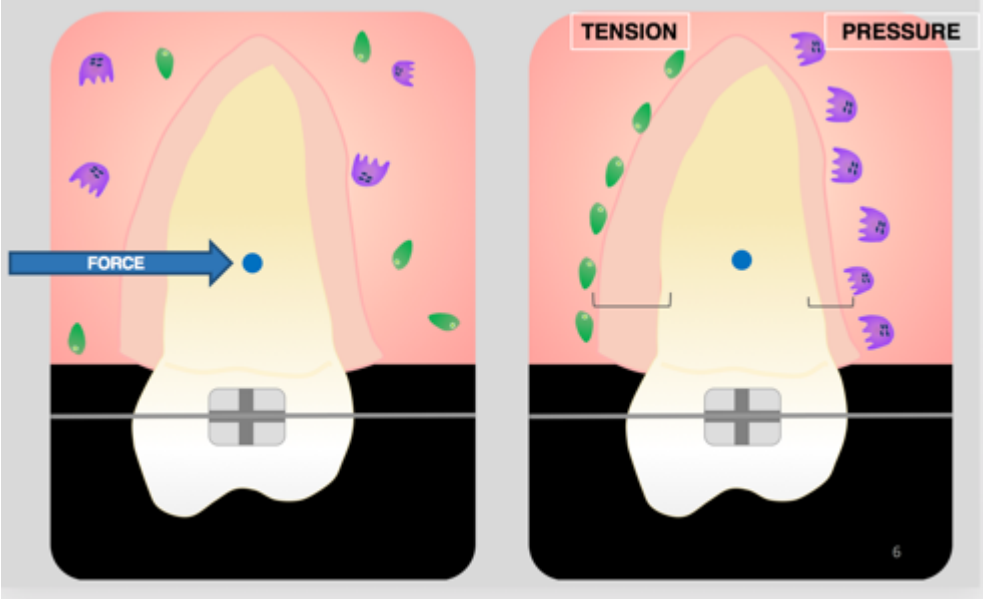

Figure 1. Bodily movement. Blue circle- center of resistance. Green cells- osteoblasts. Purple cells- osteoclasts. Left panel indicates homeostasis; right panel indicates bone remodeling.

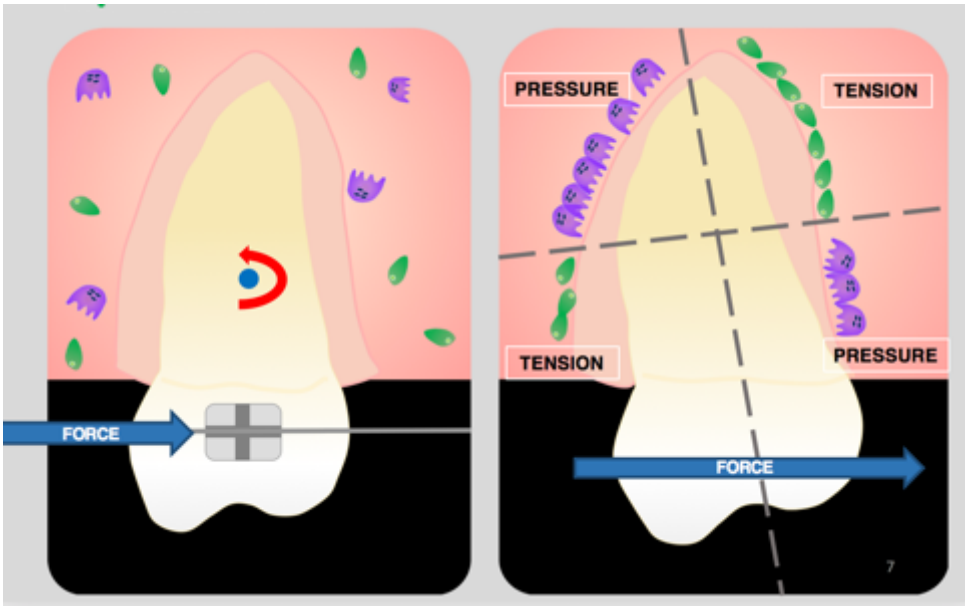

Figure 2. Tipping. Blue circle- center of resistance. Red arrow- torque. Green cells- osteoblasts. Purple cells- osteoclasts. Left panel indicates homeostasis. Right panel indicates bone remodeling patterning expected to be generated by force applied to the crown.

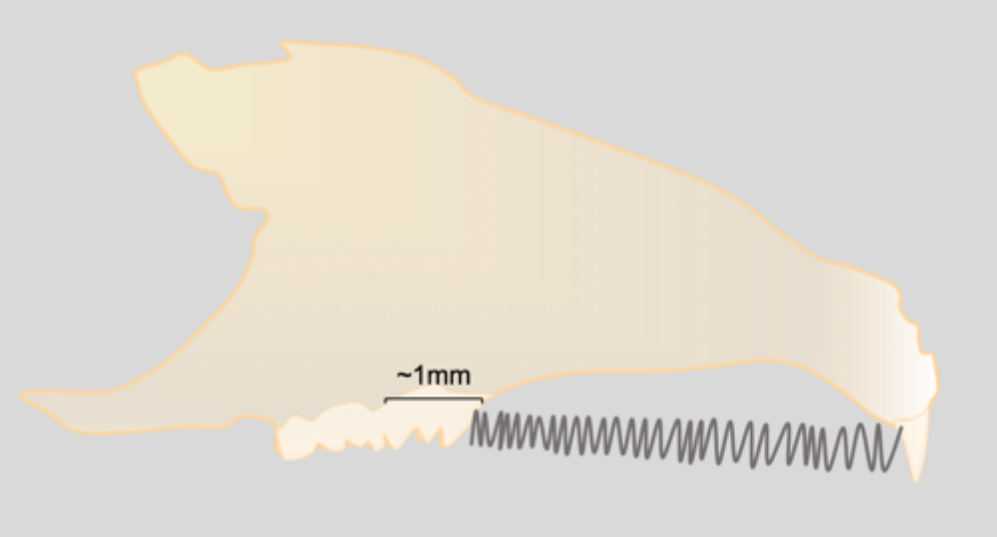

Figure 3. Orthodontic spring design. Springs are bonded to the maxillary first molar and the maxillary inscisors.

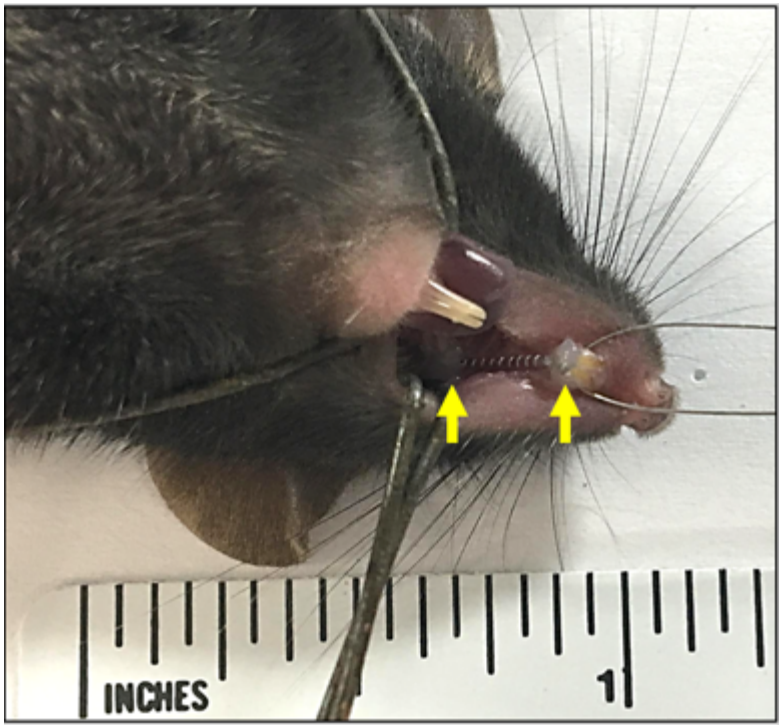

Figure 5. Bonded orthodontic springs. Yellow arrows indicate points of the spring, bonded with orthodontic resin.

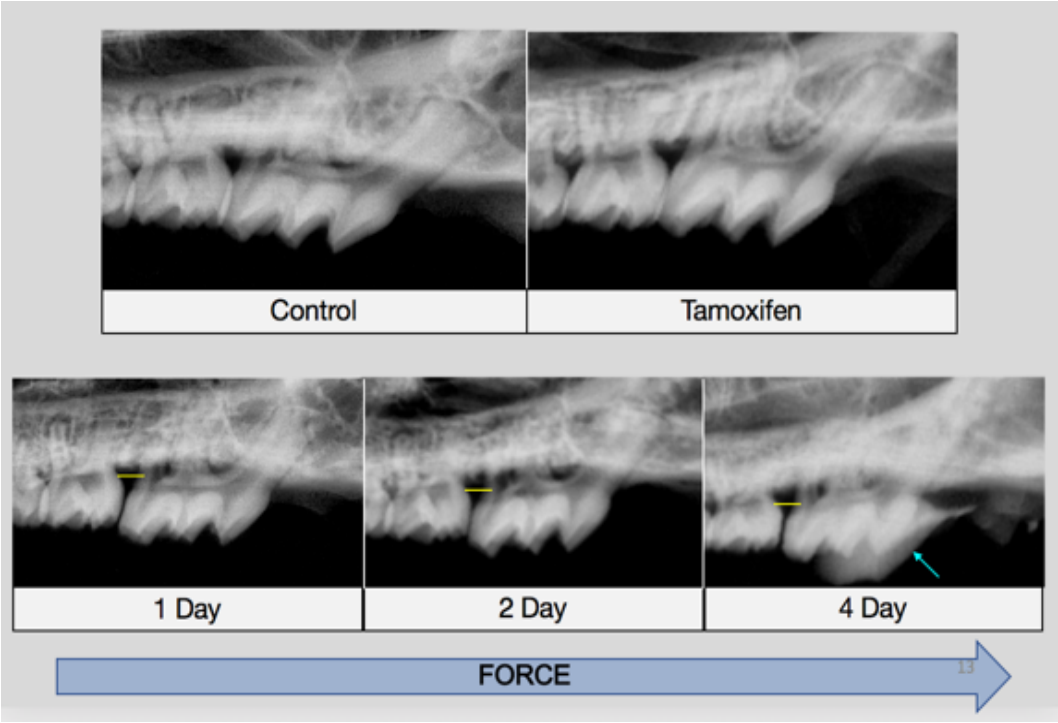

Figure 6. Radiological results. Yellow lines indicate space generated by orthodontic treatment. Blue arrow pointing to artifact of remaining orthodontic resin residue.

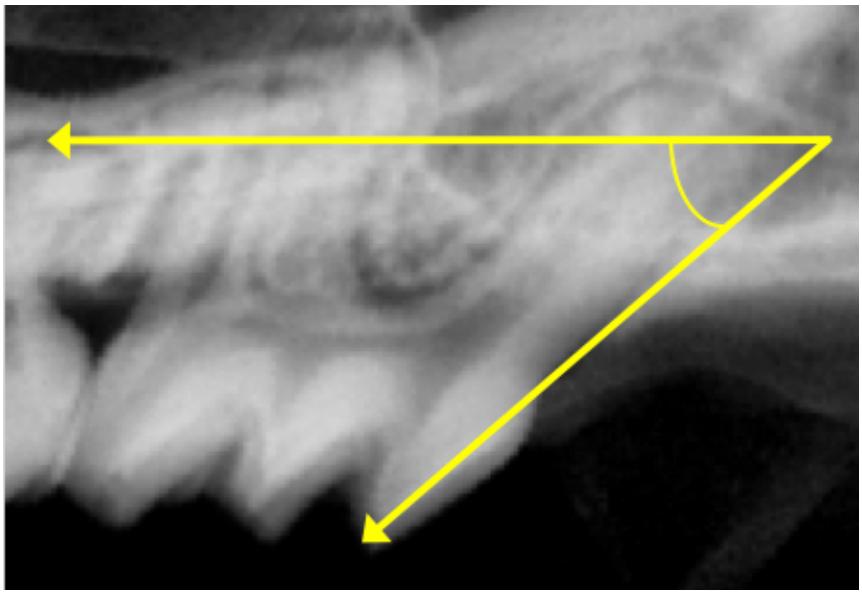

Figure 7. Angle of first molar.

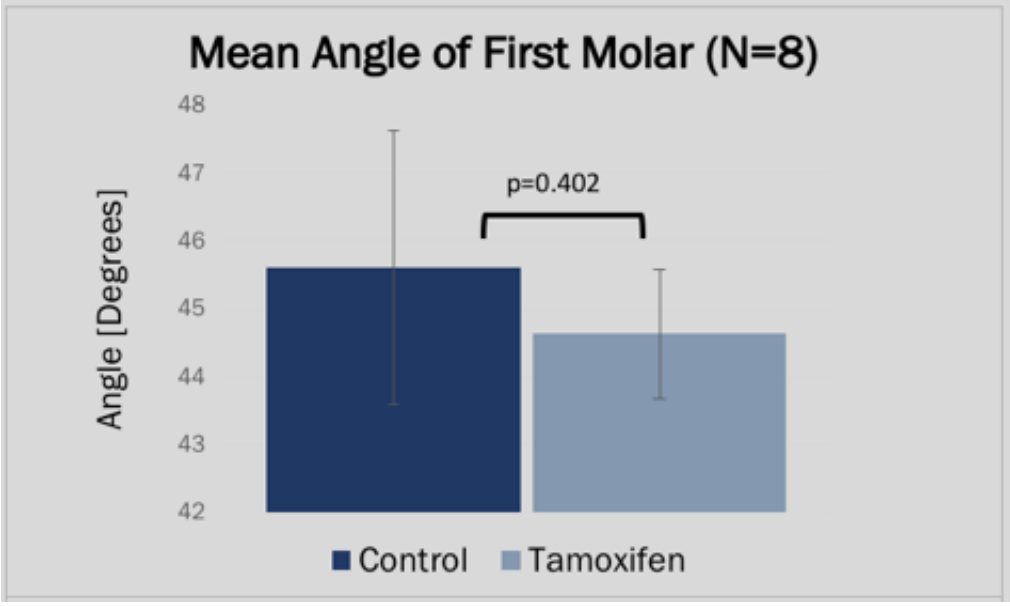

Figure 8. Difference of means of control and tamoxifen angles of first molar.

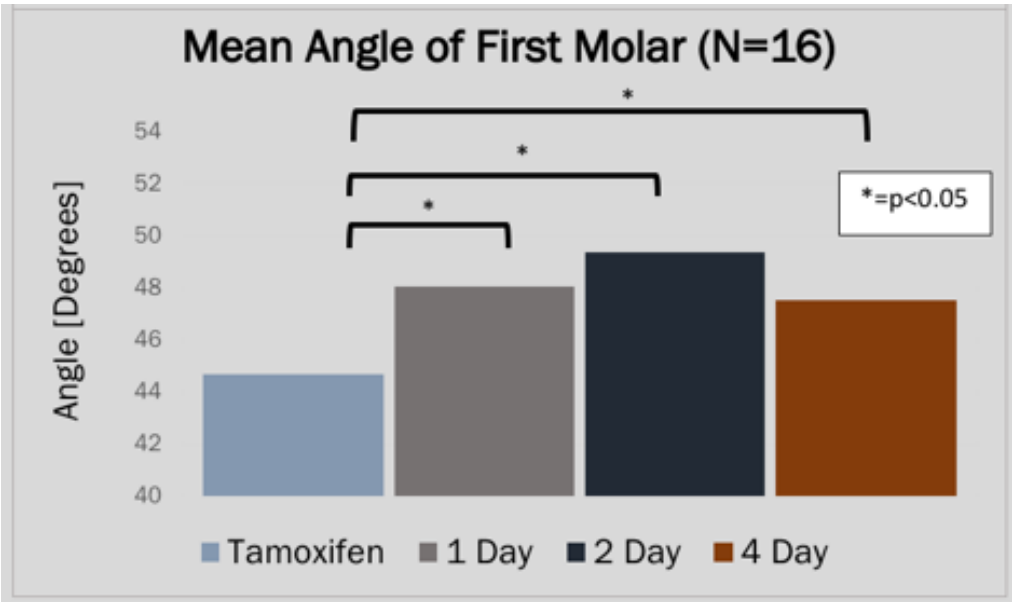

Figure 9. Difference of means comparing each orthodontic time point to tamoxifen (untreated) sample to determine if movement occurred in the orthodontic samples.

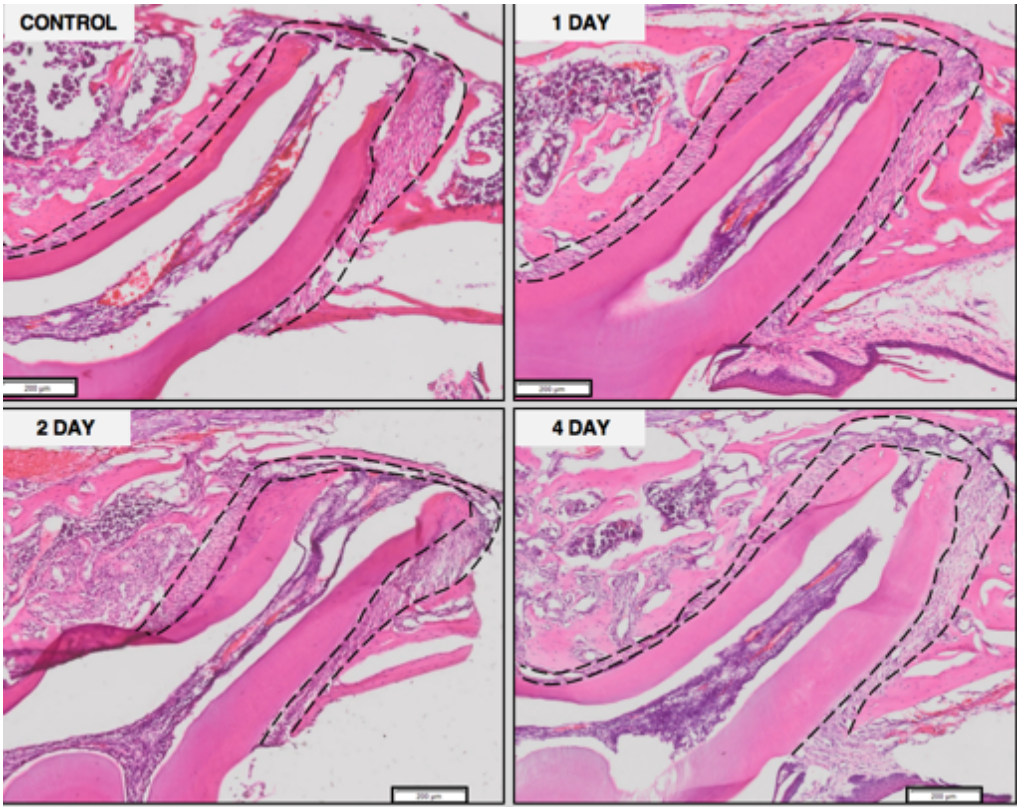

Figure 10. Histological results. HE staining clearly distinguishes between the dental tissues and the tissues of the periodontium. The periodontal ligament space is outlined.

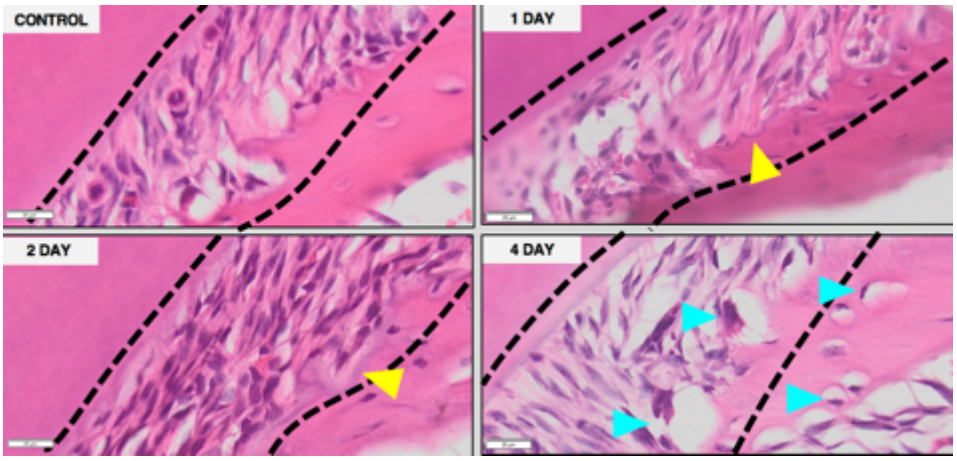

Figure 11. Histological results focusing on the mesial crown area of the alveolar crest, expected to undergo pressure. Yellow arrows indicate areas where increased acidic matrix is present when compared to the control. Blue arrows indicate osteoclast cells with resorption bays.

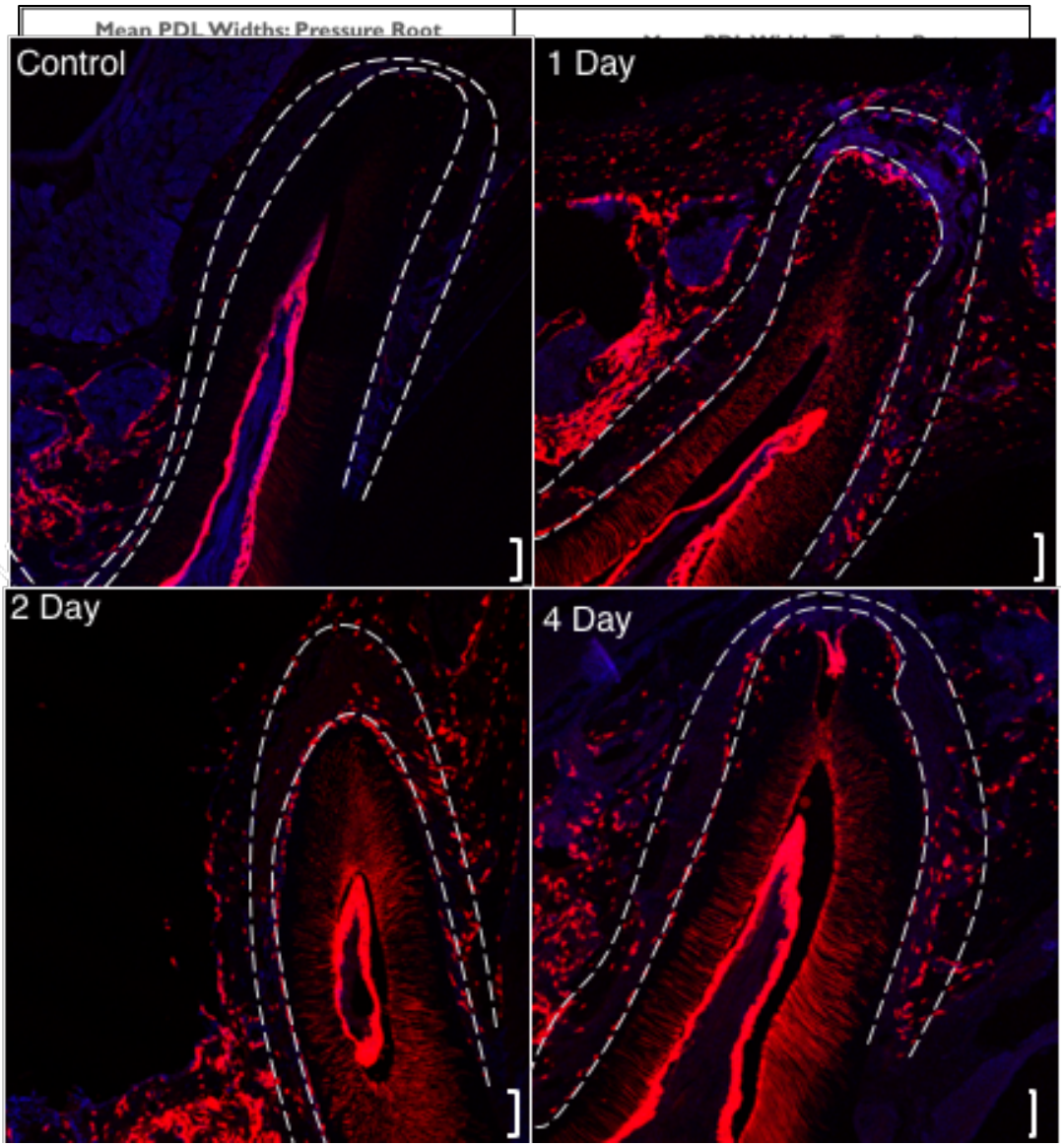

Figure 13. Characterization of the 3.2Col1a1 signal. Periodontal ligament outlined. Red- 3.2Col1a1. Blue- DAPI. Scale- 100um.

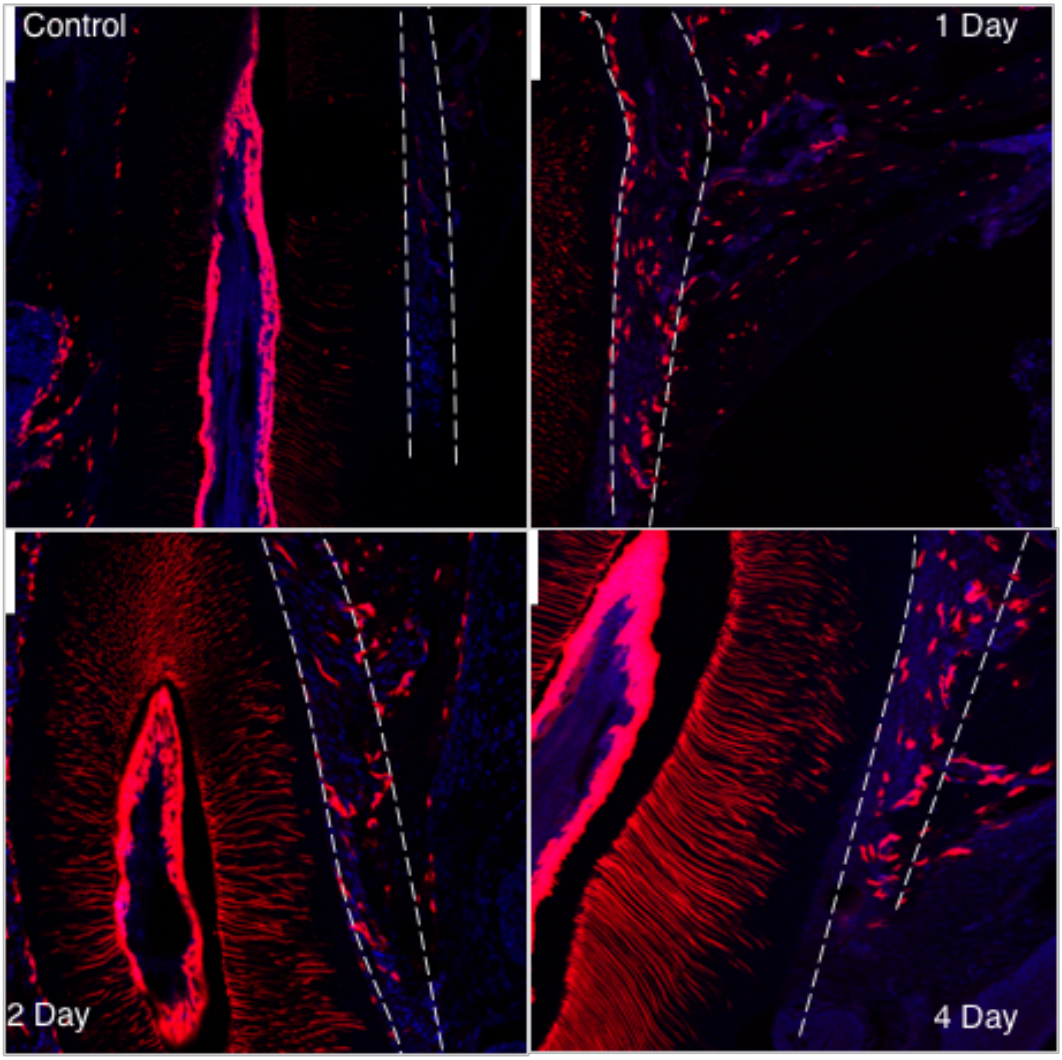

Figure 14. Mesial crown area near the alveolar crest, expected to experience pressure. Periodontal ligament outlined. Red- 3.2Col1a1. Blue- DAPI. Scale- 100um.

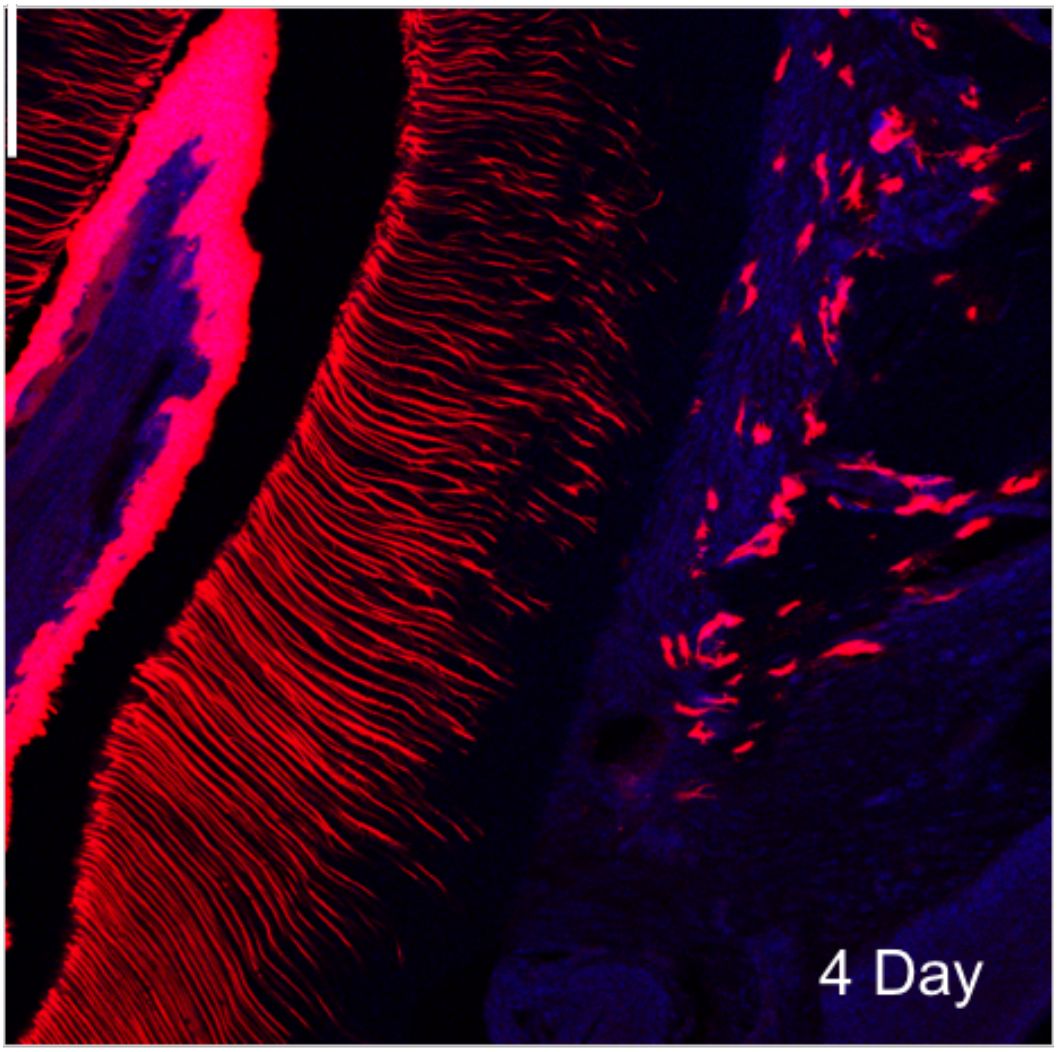

Figure 15. Odontoblast labeling in response to orthodontic tooth movement. Red- 3.2Col1a1. Blue- DAPI. Scale- 100um.

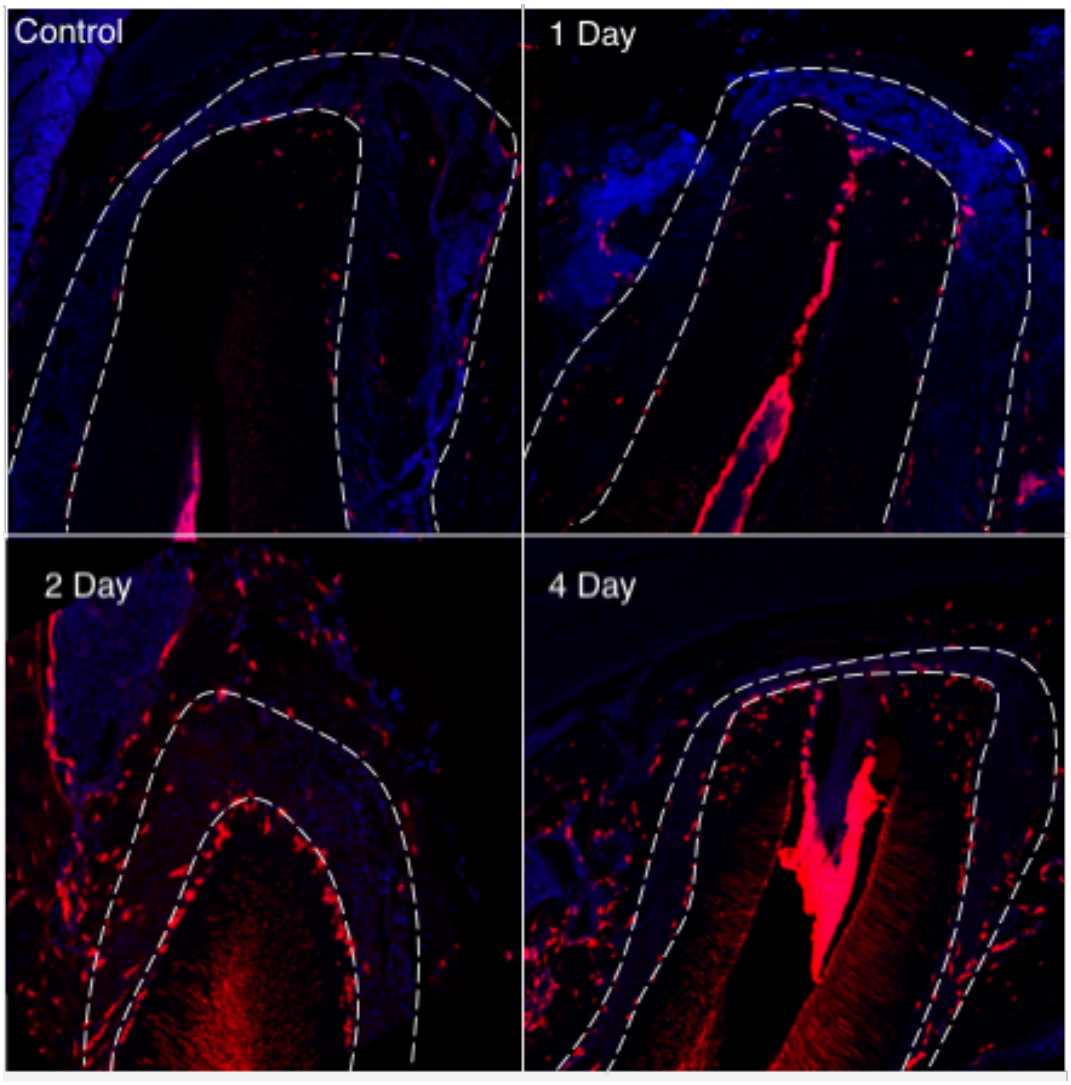

Figure 16. Root apex. Periodontal ligament outlined. Right side of root expected to undergo tension while left side is expected to undergo pressure. Red- 3.2Col1a1. Blue- DAPI.

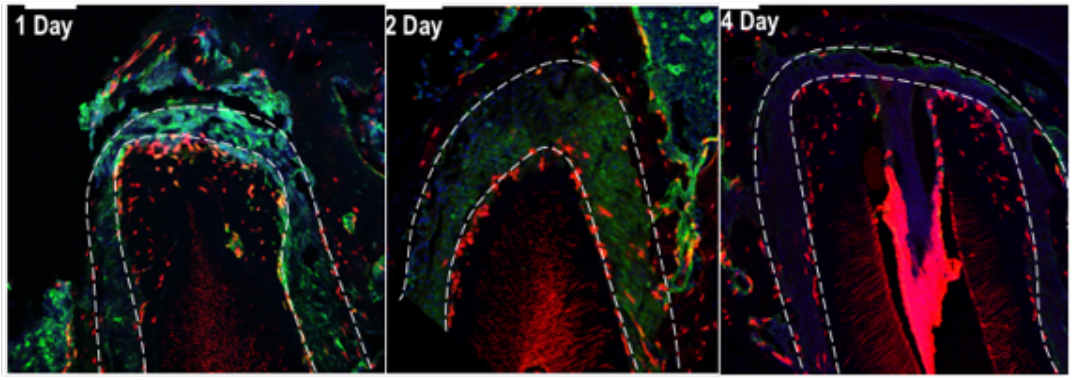

Figure 17. Ki67 immunostaining. Root apex. Right side is expected to undergo tension while left side is expected to undergo pressure. Periodontal ligament outlined. Green- Ki67. Red- 3.2Col1a1. Blue- DAPI. Scale- 100um

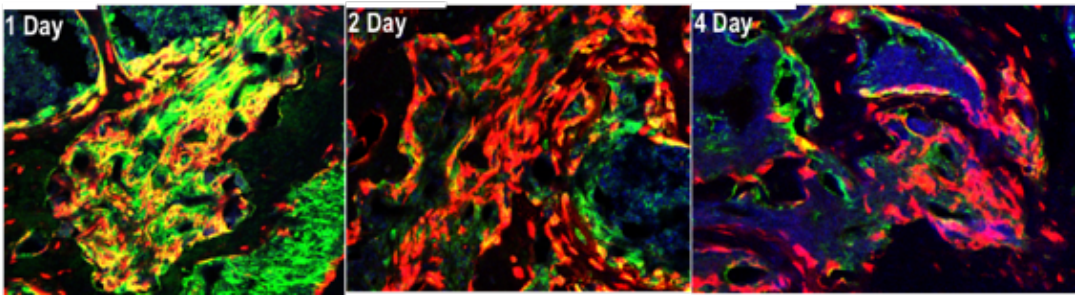

Figure 18. Ki67 immunostaining. Bone marrow. Green- Ki67. Red- 3.2Col1a1. Blue- DAPI. Scale- 100um

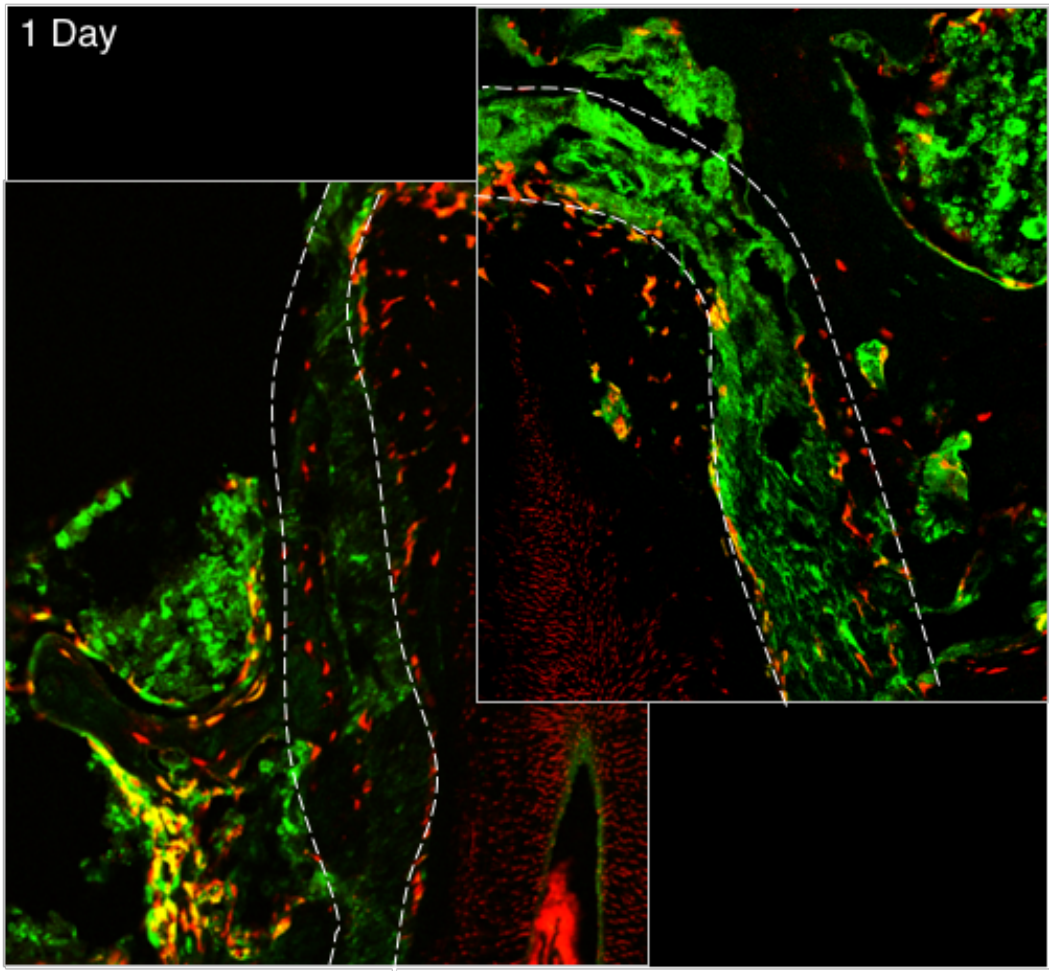

Figure 19. Ki67 immunostaining. Images manually overlayed to illustrate differences in areas of expected pressure (left) and tension (right) in terms of Ki67 expression. Note bone marrow Ki67 intensity adjacent to periodontal ligament on left panel. Green-Ki67. Red-3.2Col1a1.

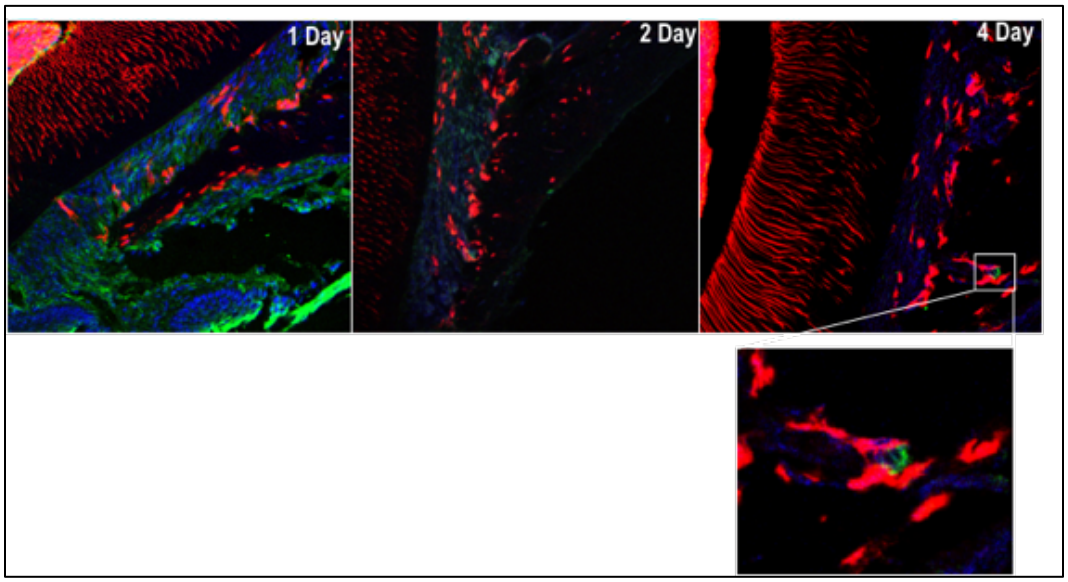

Figure 20. CathepsinK immunostaining. Mesial crown area expected to undergo pressure, near alveolar crest.Note expression in dental pulp, periosteum, and periodontal ligament. Green- CathepsinK. Red- 3.2Col1a1. Blue- DAPI.

APPENDIX B

TABLES

Treatment	Day 1	2	3	4	5	6	7
None							
Tamoxifen							
Orthodontics 4 Day			Ortho				
Orthodontics 2 Day					Ortho		
Orthodontics 1 Day						Ortho	

Table 1. Timeline design. Red- tamoxifen induction. Grey- 4-day treatment group. Navy- 2-day treatment group. Orange- 1-day treatment group. Black- sacrifice.